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TITLE: Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer: Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor

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**14. ABSTRACT**
In our preliminary study, we found that IFNγR2 has previously unknown function as an inhibitor of Bax. Bax is a key mediator of apoptosis. We found that IFNγR2 is overexpressed in prostate cancer, and we hypothesize that abnormally high level of IFNγR2 confers apoptosis resistance of prostate cancer. In this project, we will investigate the role of IFNγR2 in drug resistance of prostate cancer and explore the development of therapeutic peptide that can activate Bax-induced apoptosis in prostate cancer by inactivating IFNγR2. In Year 2, we planned to determine the presence of subtype of prostate cancer expressing high levels of IFNγR2 and Bax which is expected to be an ideal target subtype treated by IFNγR2 inhibiting strategy. We also planned to determine the effectiveness of IFNγR2 inhibition both in mouse model and cell culture model. We obtained results showing that (1) majority of prostate cancer tissue showed increased expression of IFNγR2 in comparison with normal prostate tissue, and that (2) IFNγR2 knockdown suppressed tumor growth (human cancer cells were transplanted to mouse) in nude mouse, and (3) parthenolide, a plant-derived compound, was able to decrease IFNγR2 expression in prostate cancer cells and induced cell death. We will continue our investigation to determine the mechanism of IFNγR2 over-expression in prostate cancer, and to develop technologies to induce prostate cancer cell death by targeting IFNγR2.

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## Table of Contents

**Page**

## Introduction

In our preliminary study, we identified interferon g receptor 2 (IFNγR2) as a Bax suppressor using yeast-based functional screening of Bax inhibiting proteins. Bax is a key mediator of apoptosis which is essential for chemotherapy-induced apoptosis of prostate cancer cells. We found that IFNγR2 levels is abnormally elevated in prostate cancer cell lines (both androgen-dependent and –independent cell lines). shRNA-mediated knockdown of IFNγR2 was able to increase chemotherapy-induced apoptosis rate significantly in prostate cancer cells, suggesting that IFNγR2 is an chemo-resistant factor in prostate cancer cells. Although IFNγR2 was previously known as a receptor of IFNγ which is an anti-tumorigenic cytokine, our preliminary data suggest that IFNγR2 expresses its anti-apoptosis (anti-Bax) activity independent from IFNγ and IFNγ signaling. Importantly, we found that IFNγR2 is expressed in mitochondrial membranes and ER membranes, but not on the plasma membranes of prostate cancer cells. Since we found that IFNγR2 can directly interact with Bax in vitro, we hypothesize that IFNγR2 confer apoptosis resistance of prostate cancer by directly binding and inhibiting Bax.

In this 3 years DOD Prostate Cancer Research IDEA project, the following Tasks will be examined to develop novel anti-prostate cancer therapy as well as to establish IFNγR2 as a diagnostic maker to predict the chemo-resistance of prostate cancer.

**Task 1:** To determine the mechanism of Bax inhibition by IFNγR2, and to develop anti-IFNγR2 peptide that enhances Bax activation. (Months 1-24)

**Task 2**: To identify the subtype of prostate cancer that can be effectively treated by IFNγR2-targeting technologies (Months 13-36)

**Task 3**: Determination of the mechanism of abnormal expression of IFNγR2 in prostate cancer (Months 13-36)

I the first year, Task 1 was the main part of our study and we were able to obtain information about the binding domains of IFNγR2 and Bax, as reported in the last progress report. In Year 2, experiments of Task 2 and Task 3 have started, and we obtain important results that will help us to develop new anti-prostate cancer strategy based on novel anti-apoptotic activity of IFNγR2. We are also continuing the Task 1 to identify minimum essential binding domain to develop peptide that can inhibit IFNγR2 activity to suppress Bax-mediated apoptosis.

## Body (Methods, Results and Discussion)

## Results and Discussion

**Task 2**: To identify the subtype of prostate cancer that can be effectively treated by IFNγR2-targeting technologies

### (1) Tissue Microarray experiments were performed.

We plan to perform two different approaches to investigate IFNγR2 expression patters in prostate cancer. One is to examine the correlation between IFNγR2 expression (and other anti-apoptotic proteins as well as previously known factors influencing prostate cancer behavior such as androgen receptor, Akt, or PTEN, for example) and clinical outcome using patient specimen library, and another one is to utilize commercially available tissue microarray of human prostate cancer. For the first approach, we need to obtain IRB protocol approval. As explained in section (3), we received

**Fig.1** Increased expression of INFgR2 was observed in human prostate cancer tissue microarray. (Please see the definition of cancer progression grade (Grade) and metastasis activity (TNM) in the last page of the proposal)

| No. | Position | Age | Grade | TNM | IFNγR2 | Bax | Bcl-2 |
|---|---|---|---|---|---|---|---|
| 1 | A1 | 69 | 2 | T2N0M0 | ++ | + | - |
| 2 | A2 | 69 | 2 | T2N0M0 | ± | + | - |
| 3 | A3 | 76 | 2-3 | T3N1M1b | ± ~ ++ | + | + |
| 4 | A4 | 76 | 2-3 | T3N1M1b | ++ | + | ± |
| 5 | A5 | 69 | 2 | T2N0M0 | + | - | - |
| 6 | A6 | 69 | 2 | T2N0M0 | + | - | - |
| 7 | A7 | 76 | 2-3 | T3N1M1b | ±and++ | - | - |
| 8 | A8 | 76 | 2-3 | T3N1M1b | + | ± | -and+ |
| 9 | B1 | 72 | 3-4 | T3N0M0 | + | + | +(N) |
| 10 | B2 | 72 | 3-4 | T3N0M0 | + ~ ± | + | +(N) |
| 11 | B3 | 60 | 4 | T2N0M0 | - ~ ± | + | +and- |
| 12 | B4 | 60 | 4 | T2N0M0 | ± | ± | +and- |
| 13 | B7 | 60 | 4 | T2N0M0 | ± ~ + | - | + |
| 14 | B8 | 60 | 4 | T2N0M0 | ± | ± | - |
| 15 | B5 | 72 | hyperplasia | T3N0M0 | ± ~ ++ | -and+ | ± |
| 16 | B6 | 72 | hyperplasia | T3N0M0 | ++ | -and+ | + |
| 17 | C1 | 43 | Normal | - | - | - | - |
| 18 | C2 | 43 | Normal | - | - | - | - |
| 19 | C3 | 28 | Normal | - | - | ± | ± |
| 20 | C4 | 28 | Normal | - | ± | ± | ± |
| 21 | C5 | 43 | Normal | - | - | - | - |
| 22 | C6 | 43 | Normal | - | - | - | - |
| 23 | C7 | 28 | Normal | - | - ~ ± | - | ± |
| 24 | C8 | 28 | Normal | - | - ~ ± | ± | - |

approval, and we will perform this experiment in year 3. In year 2, we examined the second strategy and examined 11 prostate cancer patient samples together with 8 normal prostate specimen as controls. The summary is presented in **Fig.1.**

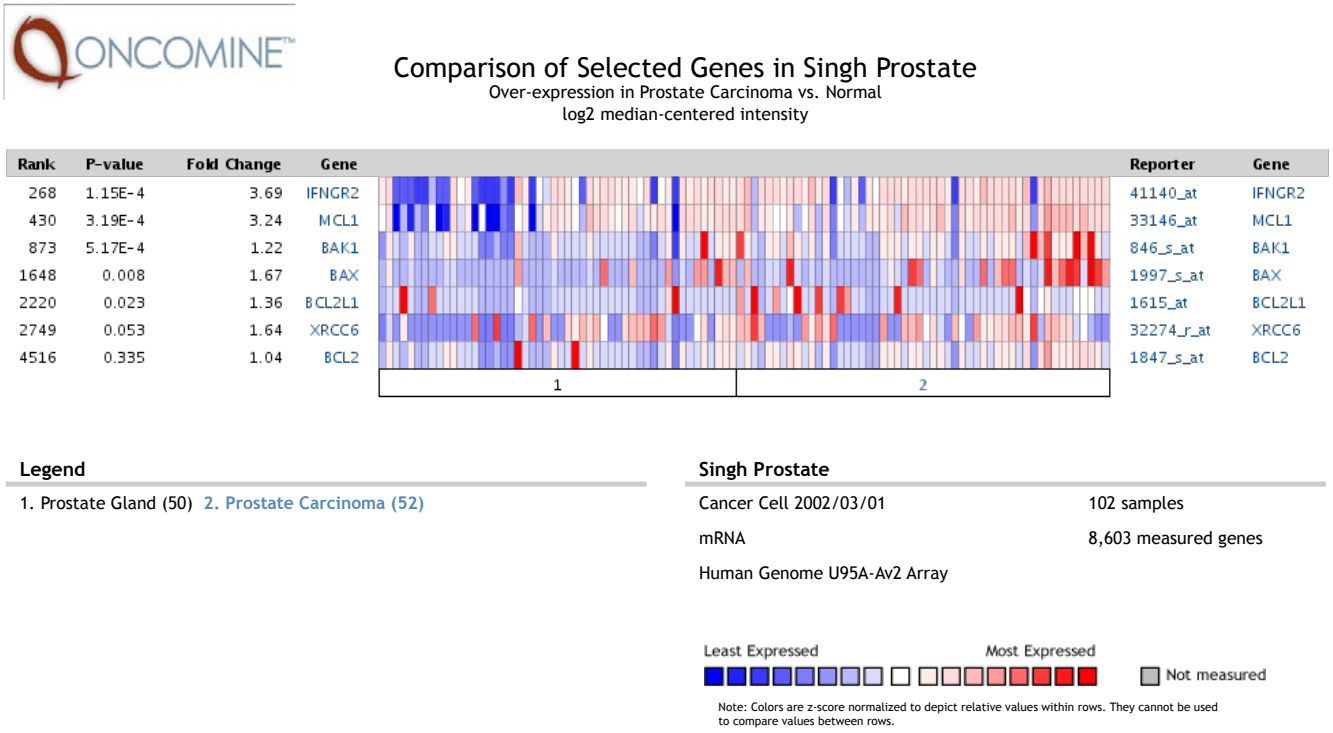
We found that majority of progressed prostate cancer specimen showed elevated expression of IFNγR2, and these cells also express Bax. Some of advanced prostate cancer (such as No. 1-4, 9, 10 and 11 Fig.1) expresses high levels of IFNγ2 as well as Bax, but no detectable Bcl-2. In this type, prostate cancer cells may suppress Bax-induced apoptosis mainly by depending on IFNγ2, and thus IFNγR2 targeting therapy is expected to work well. In the case of prostate cancer expressing both IFNγR2 and Bcl2, combinational treatment of IFNγR2 inhibitor and Bcl2 inhibitor (such as ABT-263 derivatives) may be effective. We will continue characterization of prostate cancer type by examining expression patterns of IFNγR2 and Bcl-2 family member proteins to characterize subtypes of prostate cancer that can be effective targets of IFNγR2-inhibiting therapy.

**(2) IFNγR2 expression patterns in prostate cancer were investigated by analyzing human patient database.**

Using Oncomine database (publically available gene expression profile database of human cancer patients), we found that IFNγR2 expression levels of prostate cancer are significantly higher than normal prostate as we expected. To determine the significance of IFNγR2 elevation in apoptosis-resistance of prostate cancer, we also checked the expression levels of other well-known apoptosis inhibiting proteins such as Bcl-2, Bcl-XL, and Mcl-1. Results are presented in **Fig.2**. Very

interestingly, only IFNγR2 (3.69 times increase, p<0.000115) and Mcl-1 (3.24 times increase, p<0.000319) showed significant increased expression in prostate cancer in comparison with normal prostate, but not Bcl-2 and Bcl-XL (BCLL1 in the figure). Expression of apoptosis inducer such as Bax and Bak did not show remarkable changes. These results suggest that IFNγR2 and Mcl1, but not Bcl-2 and BclXL, are the ideal targets to induce Bax/Bak-mediated apoptosis in prostate cancer. This information also suggests that Mcl1 inhibition may be also necessary to induce prostate cancer cell death when IFNγR2 inhibition is used to enhance Bax-induced apoptosis.

**Fig.2** IFNgR2 expression is higher in prostate cancer than that in normal prostate



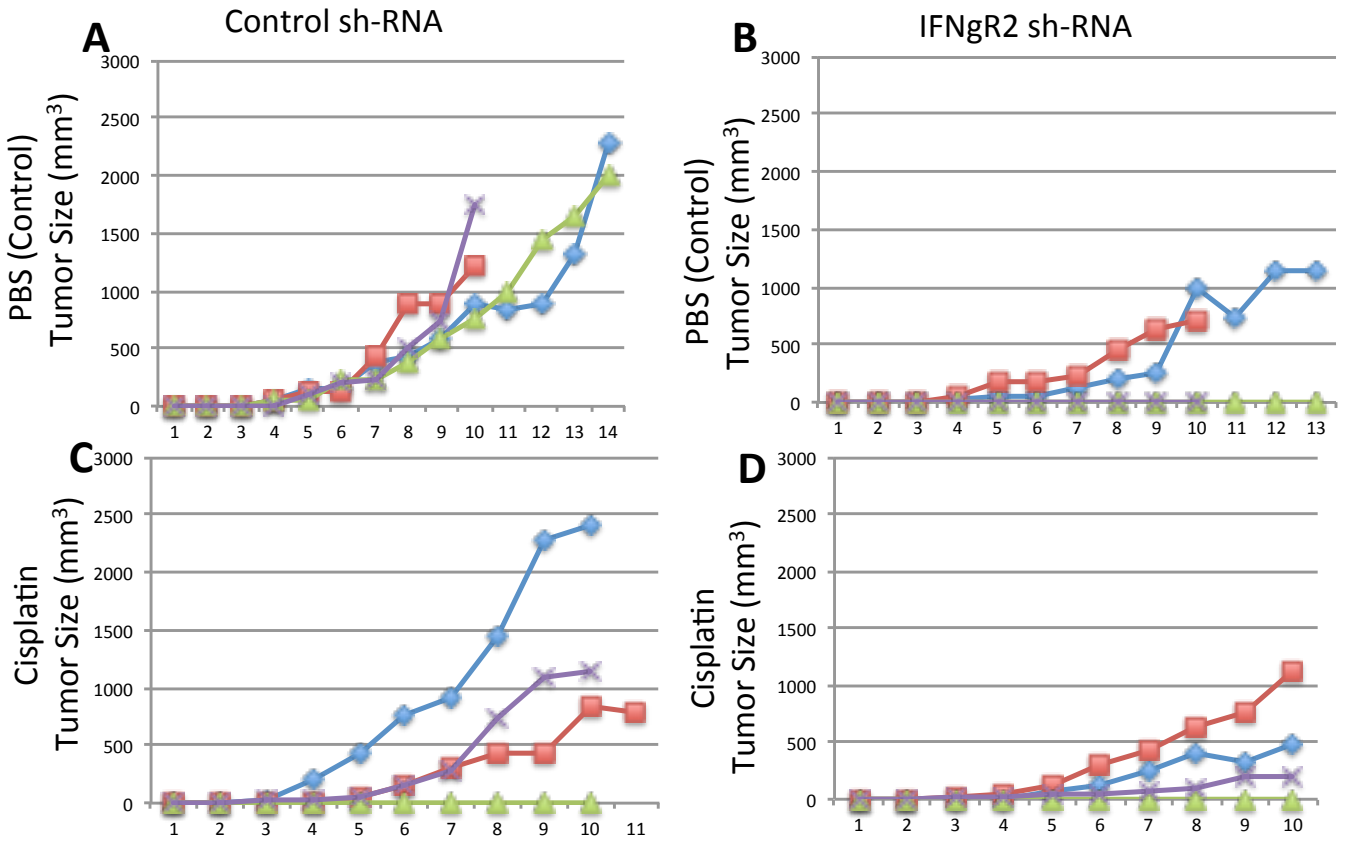

**(3) IRB protocol has been approved.**

In year 3, we are planning to further investigate the correlation of IFNγR2 expression pattern (levels and subcellular localization) and clinical outcome (survival rate/recurrence rate) using specimens and clinical treatment records in our cacner center (Case Comprehensive Cancer Center). We have already obtained IRB protocol approval for this study.

**Task 3**: Determination of the mechanism of abnormal expression of IFNγR2 in prostate cancer (Months 13-36)

**(1) Effectiveness of IFNγR2 inhibition to promote prostate cancer cell death was examined using mouse xenograph experiments.**

**Fig.3** IFNgR2 shRNA suppressed tumor growth and promoted cisplatin effects
(Each line represents each mouse treated with cancer cell and drug (or PBS))



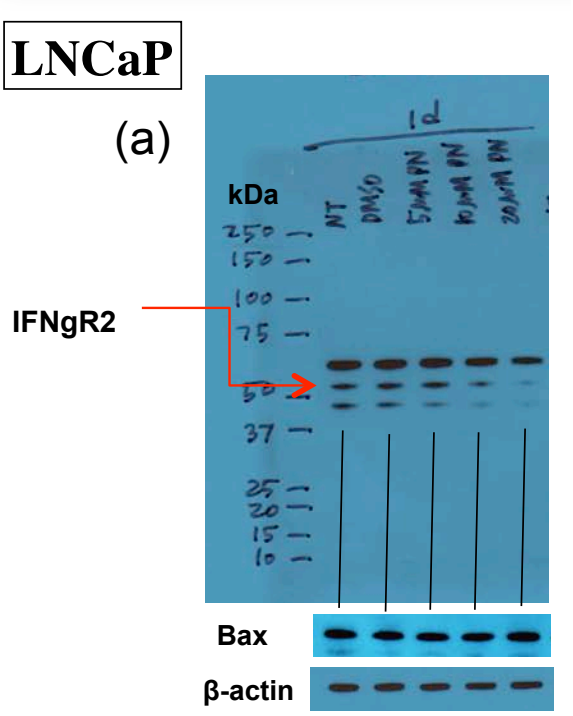

We prepared two cancer cell lines in which IFNγR2 was knocked down by shRNA. These cell lines are PC3 (human prostate cancer cell line) and A375 (human melanoma cell line). We examined A375 cell line, since we found that this cell line also express high levels of IFNγR2 and this cell line can be used as a model cancer cell line to determine the effectiveness of anti-IFNγR2 therapy.

We injected one million cells of cancer cells to each nude mouse, and docetaxel (1mg/kg) and cisplatin (5mg/kg) were treated every week to mice. In PC3 cell line experiments, more than a half of docetaxel-treated mice (2-3 out of 4 mice) were dead within two weeks of treatment, therefore we were not able to obtain reportable result. We are planning to repeat this experiments using lower dose of docetaxel. In the case of cisplatin-treated A375 experiments, we were able to obtain preliminary results to determine the effects of IFNγR2 knockdown. IFNγR2 knockdown was able to slow down the growth of tumor (Fig.3 panel B) in comparison with control shRNA expressing cells (Fig.3 panel A). Tumor growth after cisplatin-treatment was also suppressed by shRNA-mediated IFNγR2 knockdown (Fig.3 panel D) in comparison with control (Fig.3 panel C), though whether cisplatin-induced cell death was “enhanced” is not yet clear, since IFNγR2 shRNA alone (without cisplatin) showed significant suppression of tumor growth (Fig.3 panel A vs B). Further experiments will be performed to determine whether IFNγR2 knockdown can enhance chemotherapy-induced cell death in prostate cancer.

**(2) Effects of NFkB inhibitor (Parthenolide) to suppress IFNγR2 expression**

To develop technologies targeting IFNγR2, we proposed to determine the effectiveness of currently available drugs that is predicted to decrease IFNγR2 expression in prostate cancer. Since previous studies have shown that NFkB is one of transcription factors that stimulate IFNγR2 gene expression, we proposed to determine the effects of NFkB inhibitor. Parthenolide is a plant-derived compound which is known to inhibit NFkB activity. In our preliminary study, we found that parthenolide was able to decrease IFNγR2 expression in PC3 prostate cancer cells. In this study, we examined another standard prostate cancer cells, LNCaP cell line (Fig.4). We found that IFNγR2 expression was suppressed by parthenolide (from 5 uM) within 1 day after the treatment (Fig. 4 shows the result of 1 day treatment). Importantly, Bax expression was not decreased by this treatment, suggesting that parthenolide can stimulate Bax-mediated cell death by decreasing Bax inhibitor, i.e. IFNγR2. In our preliminary study, we confirmed that parthenolide, in fact, induces apoptosis in both PC3 and LNCaP cell lines. In year 3, we will examine whether parthenolide can enhance apoptosis triggered by other anti-cancer drugs.

**Fig.4** Parthenlide treatment decreased IFNgR2 expression without changes in Bax expression




**Methods:**

*Immunohistochemistry of human prostate cancer tissue microarray.*

Human prostate cancer tissue microarray was purchased from BioMax (Maryland, USA). Immunohistochemistry of IFNγR2, Bax, and Bcl-2 were performed by the standard methods explained in detail in Abcam website (http://www.abcam.com/index.html?pageconfig=resource&rid=13046).

Antibodies used in these experiments are: IFNγR2 (Abcam, #ab77246), Bax (BD Pharmingen #554104), and Bcl-2 (BD Pharmingen #514202).

*Definition of tumor grade in Fig.1*

The Grade 1-3 in Pathology Diagnosis is equivalent to well-differentiated, moderately-differentiated or poorly differentiated, respectively, under microscope.

**Grade 1 or well-differentiated:** Cells appear normal and are not growing rapidly.
**Grade2 or moderately-differentiated:** Cells appear slightly different than normal.
**Grade3 or poorly differentiated:** Cells appear abnormal and tend to grow and spread more aggressively.
**Grade 4 or undifferentiated:** *(for certain tumors), features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

**TNM grading:**

**T - Primary tumor**
Tx - Primary tumor cannot be assessed
T0 - No evidence of primary tumor
Tis - Carcinoma in situ; intraepithelial or invasion of lamina propria
T1 - Tumor invades submucosa
T2 - Tumor invades muscularis propria
T3 - Tumor invades through musclaris propria into subserosa or into non-peritonealized pericolic or perirectal tissues
T4 - Tumor directly invades other organs or structures and/or perforate visceral peritoneum

**N - Regional lymph nodes**
Nx - Regional lymph nodes cannot be assessed
N0 - No Regional lymph node metastasis
N1 - Metastasis in 1 to 3 regional lymph nodes
N2 - Metastasis in 4 or more regional lymph nodes

**M - Distant Metastasis**
Mx - Distant metastasis cannot be assessed
M0 - No distant metastasis
M1 - Distant metastasis

*Cell culture and cell lysate preparation for Western blot*

PC3 and LNCaP cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and1% penicillin/streptomycin. To determine the effects of parthenolide, cells were cultured in the presence of various concentration of parthenolide (5, 10, or 20 ug/ml) for 1 day. Cell lysates were prepared by solubilizing cell pellets using 1% NP40 containing HEPES buffer. Insoluble fraction was removed by centrifuge separation (14k rpm for 20n min at 4C). For the analysis of protein expression, cell lysates containing 10 ug protein were used. SDS-PAGE was performed by using 4-20% gradient gel, and immuno-detection was performed by ECA Chemical luminescence detection kit (Amersham).

*Mouse xenograph experiments*

One million cells of cancer cells (PC3 and A375) were subcutaneously injected to nude mice. One week later, docetaxel (1 mg/kg) or cisplatin (5 mg/kg) were administered (i.p. injection) once a week,

for 4-5 weeks. When tumor size reaches 10% of mouse body, experiments were stopped, and mice were euthanized for tumor size analysis.

**Key Research Accomplishment**

1. Existence of subtypes of prostate cancer expressing high levels if IFNγR2 was confirmed by using human prostate cancer tissue micro array and publically available gene expression data base.
2. IRB protocol to determine the correlation between IFNγR2 expression levels and clinical outcome has been approved.
3. Effectiveness of IFNγR2 inhibition to enhance anti-cancer drug effects was confirmed by mouse xerograph experiments.
4. Effect of plant-derived NFkB inhibitor, parthenolide, to decrease expression levels of IFNγR2 in prostate cancer was found.

**Reportable Outcome**

We will present this study in the international meeting of interferon held in Melbourne, Australia, in November, 2014. Actually, PI was invited as a selected speaker in this meeting. All the key research accomplishments are reportable results for the future publication. To publish our findings, we think that further study identifying the minimum element for the binding is necessary. We will continue our investigation to prepare manuscript for submission.